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DNA Sequencing and Homologous Expression of a Small Peptide Conferring Immunity to Gassericin A, a Circular Bacteriocin Produced by *Lactobacillus gasseri* LA39▽

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Gassericin A, produced by *Lactobacillus gasseri* LA39, is a hydrophobic circular bacteriocin. The DNA region surrounding the gassericin A structural gene, *gaaA*, was sequenced, and seven open reading frames (ORFs) of 3.5 kbp (*gaaBCADITE*) were found with possible functions in gassericin A production, secretion, and immunity. The deduced products of the five consecutive ORFs *gaaADITE* have homology to those of genes involved in butyrivibriocin AR10 production, although the genetic arrangements are different in the two circular bacteriocin genes. GaaI is a small, positively charged hydrophobic peptide of 53 amino acids containing a putative transmembrane segment. Heterologous expression and homologous expression of GaaI in *Lactococcus lactis* subsp. *cremoris* MG1363 and *L. gasseri* JCM1131^T, respectively, were studied. GaaI-expressing strains exhibited at least sevenfold-higher resistance to gassericin A than corresponding control strains, indicating that *gaaI* encodes an immunity peptide for gassericin A. Comparison of GaaI to peptides with similar characteristics found in the circular bacteriocin gene loci is discussed.

Bacteriocins are antimicrobial peptides that act primarily against related bacterial species. The classification of bacteriocins remains controversial. Here, we use the classification of Maqueda et al. (30): class I (lantibiotics); class II (nonlantibiotics) with subclasses IIa (antilisteral pediocin-like bacteriocins), IIb (two-peptide bacteriocins), and IIc (leaderless bacteriocins); class III (large heat-labile bacteriocins); and class IV (circular bacteriocins linked at the N- and C-terminal amino acids).

Nine class IV circular bacteriocins have been reported to date. They can be further divided into two major groups by using their primary structures, biochemical characteristics, and genetic arrangements. One group is the family of enterocin AS-48 (32), the first circular bacteriocin described (in 1994), which includes circularin A (25) and uberolysin (40). The other group is the family of gassericin A (19, 21), the second bacteriocin found (in 1998), which includes acidocin B (28), reutericin 6 (with a primary structure 100% identical to that of gassericin A) (22, 23), butyrivibriocin AR10 (17), and carnocyclin A, from *Carnobacterium maltaromaticum* UAL307 (33). The lantibiotic-like subtilosin A produced by *Bacillus subtilis* subsp. *subtilis* strain 168 (24) is an orphan member of the class IV bacteriocins. The gassericin A family of bacteriocins have been isolated from various bacterial species in several countries, suggesting the bacteriocin genes may be associated with transferable genetic elements.

The bacteriocins of lactic acid bacteria (LAB) and bacteriocin-producing LAB strains isolated from foods are promising food preservative candidates, and strains of human origin are expected to be probiotics that could help to prevent the growth of harmful bacteria in food and the human intestine. *Lactobacillus gasseri* belongs to the *Lactobacillus acidophilus* group of LAB, which are natural inhabitants of the human intestinal tract (35), and many *L. gasseri* strains have been shown to produce bacteriocins (16, 20). Gassericin A was produced by *L. gasseri* LA39 isolated from the feces of a human infant; it has bactericidal activity against the food-borne pathogens *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* (16). Recently, using proteose peptone, some strains of *L. gasseri* containing LA39 were successfully cultured in reconstituted skim milk and cheese whey, where *L. gasseri* LA39 produced gassericin A; these low-cost, safe media could be used to improve the safety of biopreservation (1). Gassericin A has been purified and characterized, and its structural gene (*gaaA*) has been cloned and sequenced (21, 22). Determination of the complete chemical structure of gassericin A showed that the bacteriocin belongs to class IV and consists of 58 amino acid residues linked at the N and C termini (19). Little is known about the mechanisms of secretion and circularization of gassericin A and immunity to the circular bacteriocin.

Here, we sequenced six genes surrounding *gaaA* thought to be related to production of and immunity to gassericin A and examined the homologous and heterologous expression of a

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
L. gasseri		
LA39	Gassericin A producer = JCM11657	Laboratory collection
JCM1131^T	Bacteriocin indicator = ATCC 33323^T	JCM[b]
E. faecalis JH2-2	Plasmid-free derivative of *E. faecalis* JH-2	17
L. lactis subsp. *cremoris* MG1363	Plasmid-free derivative of NCDO712	11
E. coli DH5α	Plasmid free	12
Plasmids		
pIL253-P32	Em^r; pIL253 derivative with P_{32} promoter	26
pCR2.1-TOPO	Amp^r Km^r	Invitrogen
pCR2.1-LG45	Amp^r Km^r	This study
pGAI	Em^r; pIL253-P32 derivative carrying *gaaI*	This study

[a] Amp^r, ampicillin resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance.
[b] JCM, Japan Collection of Microorganisms.

small hydrophobic peptide, GaaI; we found that *gaaI* is an immunity gene providing protection against gassericin A.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. The gassericin A producer, *L. gasseri* LA39 (JCM11657), isolated in our laboratory from the feces of a 4-month-old infant (23), and the non-bacteriocin producer *L. gasseri* JCM1131^T (ATCC 33323^T) (2) were grown at 37°C in MRS broth (Difco Laboratories, Detroit, MI). M17 broth (Difco) with 0.5% (wt/vol) glucose (GM17) was used for the cultures of *Lactococcus lactis* subsp. *cremoris* MG1363 at 30°C and *Enterococcus faecalis* JH2-2 at 37°C. *Escherichia coli* DH5α was cultured for 16 h in tryptone-yeast broth with vigorous agitation (250 rpm) at 37°C. Broth agar and soft-agar media contained 1.5% (wt/vol) and 0.7% (wt/vol) agar (agar no. 1; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), respectively. To select and maintain transformants, ampicillin (Sigma, Zwijndrecht, The Netherlands) was used at 100 μg/ml for *E. coli* and erythromycin (Sigma) was used at 5 μg/ml for *L. gasseri*, *L. lactis* subsp. *cremoris*, and *E. faecalis*.

Nucleotide sequencing. The nucleotide sequence surrounding *gaaA* was determined by primer walking using the total DNA of *L. gasseri* LA39 as the template. PCR fragments were purified using the High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and were sequenced either directly or after being subcloned into pCR2.1-TOPO. DNA sequencing was performed using the dideoxy chain termination method with a Prism 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Japan Ltd.) or using T7 primers with the AlfII system (Amersham Pharmacia Biotech) according to the protocols of the manufacturers.

Computational analyses. Open reading frames (ORFs) were identified using the Glimmer 2.0 program (6) and/or GENETYX-MAC software (Software Development, Tokyo, Japan). Homology searches were performed using the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html) in the DDBJ databases. Transmembrane regions in peptides were deduced using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) (13).

Cloning methods and materials. Molecular cloning techniques were performed essentially as described by Sambrook et al. (36). The restriction enzymes, T4 DNA ligase and Expand DNA polymerase, were obtained from Roche Diagnostics GmbH and used as described by the manufacturer. Plasmid DNA was introduced into *L. lactis* subsp. *cremoris* MG1363 and *L. gasseri* JCM1131^T using the electroporation methods described by Holo and Nes (14) and Luchansky et al. (29), respectively. *E. faecalis* JH2-2 was grown in the presence of 8% (wt/vol) glycine to prepare electrocompetent cells (http://www.enterococcus.ouhsc.edu/electroporation.asp) (37). After transformation, *L. lactis* subsp. *cremoris* MG1363 and *E. faecalis* JH2-2 were inoculated onto GM17 agar containing 0.5 M sucrose and erythromycin, and *L. gasseri* JCM1131^T was inoculated onto MRS agar containing erythromycin. Plasmids from these strains were prepared according to the method of Birnboim and Doly (3).

Cloning of the hetero- and homodeterminants carrying the immunity peptide for gassericin A. The *gaaI* region was amplified using Expand DNA polymerase with the primers 5′-ACGCGTCGACTTGTGCAGTACGTTATTTAAG-3′ and 5′-GCTCTAGACATCTACCCTTTATCCTTTGTTAC-3′. The SalI and XbaI sites are underlined in the primer sequences. The PCR product was cloned into pCR2.1-TOPO with the Topo TA cloning kit (Invitrogen, Breda, The Netherlands) to create pCR2.1-LG45 using *E. coli* DH5α. After digestion of pCR2.1-LG45 with SalI and XbaI, the *gaaI* fragment was ligated into pIL253-P32 digested with the same enzymes. The ligation mixture was used to transform *E. faecalis* JH2-2. The resultant plasmid, designated pGAI, was introduced into *L. lactis* subsp. *cremoris* MG1363 and *L. gasseri* JCM1131^T.

Bacteriocin tolerance assay. Bacteriocin tolerance was assayed using the agar well diffusion method. Briefly, the MRS culture supernatant of *L. gasseri* LA39 containing gassericin A and the gassericin A purified as described previously (23), were mixed with 50 mM sterile sodium phosphate buffer (pH 6.8) and 60% (vol/vol) 2-propanol, respectively, and then serially diluted as 1/*n* (*n* represents integrals from 1 to 32). Appropriate broth agar plates (4 mm thick and 90 mm in diameter) were overlaid with a soft-agar lawn (10 ml) inoculated with a diluted

TABLE 2. Deduced peptides and proteins derived from *gaa*

ORF	Length (amino acids)	MW	pI	Function	Best homolog [identities (no. of amino acids, %)]	Localization (TMS no.)[a]
gaaB	174	20,278	9.6	Unknown	Acidocin B: unnamed protein product (101/103, 98)	Membrane (5)
gaaC	60	7,291	9.6	Unknown	Acidocin B: unnamed protein product (60/60, 100)	Membrane (2)
gaaA	91	9,285	9.4	Gassericin A precursor	Acidocin B: bacteriocin (89/91, 98)	Membrane (2)
gaaD	162	18,344	6.9	Unknown	Acidocin B: unnamed protein product (111/113, 98)	Membrane (4)
gaaI	53	6,135	11.1	Immunty peptide	No hit	Membrane (1)
gaaT	226	25,126	4.7	ATP-binding protein	Transporter [*Clostridium tetani* E88] (77/212, 36; positives, 131/212, 61)	Soluble (none)
gaaE	212	23,665	9.5	Accessory protein	ABC-type transport system [*Clostridium difficile* QCD-32g58] (53/214, 24; positives, 111/214, 51)	Membrane (6)

[a] Deduced using the SOSUI program.

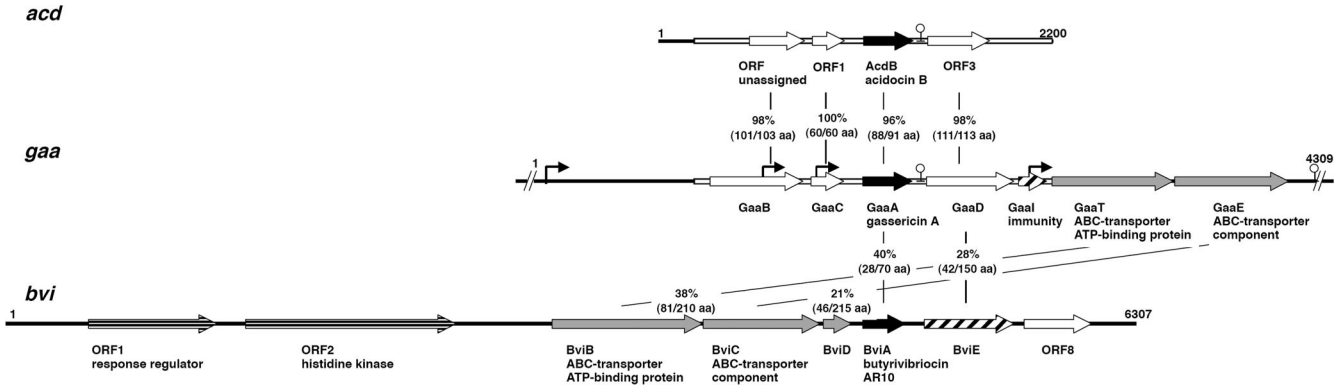

FIG. 1. Comparison of the *gaa*, *acd*, and *bvi* loci. The protein names and identified or proposed functions are shown. The structural genes *gaaA*, *acdB*, and *bviA* are represented by black arrows; the immunity genes *gaaI* and *bviE* are represented by hatched arrows; the transport genes *gaaT*, *gaaE*, *bviB*, *bviC*, and *bviD* are represented by shaded arrows; and the regulation genes of ORF1 and ORF2 in *bvi* are represented by horizontally striped arrows. The open boxes in *gaa* and *acd* indicate that the regions are 98.4% identical. Predicted promoters and terminators are represented by angled arrows and omega-like symbols, respectively.

overnight culture of each of the indicator strains at 1% (vol/vol). Wells (5 mm in diameter) were cut from the plates, and 65 μl of the serially diluted bacteriocin sample was added to each well. The minimum inhibitory dilution of the bacteriocin against the indicator strains was measured to determine the relative resistance to gassericin A. The experiments were performed in triplicate.

Nucleotide sequence accession number. The 4,100-bp DNA sequence containing the seven *gaa* genes is deposited in the DDBJ, EMBL, and GenBank databases under accession number AB007043.

RESULTS AND DISCUSSION

The nucleotide sequence of an approximately 20-kbp region surrounding the structural gene of gassericin A, *gaaA*, was sequenced by primer walking using the total DNA of *L. gasseri* LA39. Using computer analyses, seven ORFs, including *gaaA*, putatively related to gassericin A production were deduced in a 3.5-kbp region and designated *gaaBCADITE* (Table 2 and Fig. 1). ORFs outside of the 3.5-kbp region did not show similarity to known bacteriocin-related genes. *gaaI* encodes a peptide of 53 amino acid residues. Expression studies have shown that GaaI is an immunity peptide (described below). GaaT contains an ABC transporter nucleotide-binding domain (cd00267 ABC_ATPase) and is homologous to the putative ABC transporter ATP-binding proteins found in several species of *Clostridium* and *Bacillus*. GaaE is a putative membrane protein with six transmembrane regions and shows similarity to several proteins annotated as putative permeases. *gaaT* and *gaaE* may encode the exporter of gassericin A during its production process and/or may function as the auxiliary immunity mechanism, as is observed in several bacteriocin genes, such as the enterocin AS-48 gene (7, 31). *gaaB*, *gaaC*, and *gaaD* may be membrane associated, but we were unable to speculate about their functions using the bioinformatics approach. Potential promoter sequences were predicted in the *gaa* region (Fig. 1). Interestingly, most of the putative promoters are located within the coding regions of the *gaa* genes. Terminators were predicted downstream of *gaaA* and *gaaE* (Fig. 1) with ΔG values of −15.8 kcal and −9.0 kcal, respectively, but this was not determined for the other *gaa* genes.

Some of the seven *gaa* genes are similar to other circular-bacteriocin genes, especially to the acidocin B genes from *L. acidophilus* M46 (28) and the butyrivibriocin AR10 genes from the ruminal bacterium *Butyrivibrio fibrisolvens* AR10 (14) (Fig. 1). The closest comparison is to the acidocin B gene locus (*acd*), where approximately 2 kb of the reported 2,200-bp sequence of the *acd* locus is 98.4% identical to *gaa* (Fig. 1). *gaaC* and *gaaA* are almost identical to ORF1 and *acdB* in the *acd* locus, respectively. ORF3 of *acd* may be a truncation of *gaaD*. An ORF encoding 103 amino acids but unassigned in the reference was found upstream of *acd* ORF1 (Fig. 1). The newly identified ORF lacks a ribosome binding site but shows 98% amino acid identity to the C terminus of GaaB. Further, three fragmented traces of *gaaI* are found in the *acd* sequence. These ORF truncations in *acd* are caused by a number of frameshifts. The *gaa* region showed lower DNA homology but still significant genetic similarity to the putative circular butyrivibriocin

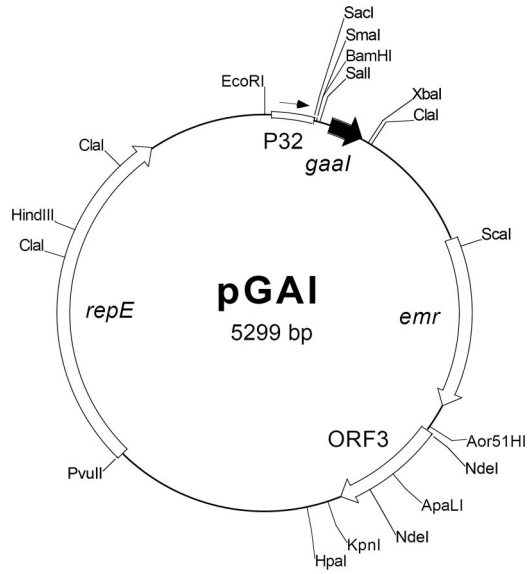

FIG. 2. Plasmid map of the *gaaI* expression vector pGAI. P32, the lactococcal constitutive promoter (39); *gaaI*, the putative gassericin A immunity gene; *emr*, the erythromycin resistance gene; ORF3, an uncharacterized ORF in the vector pIL253; and *repE*, the replication protein E gene.

TABLE 3. Minimum inhibitory dilutions for the MRS culture supernatant of *L. gasseri* LA39 and purified gassericin A against the constructed strains

Tested strain	Minimum inhibitory dilution (fold)[a]	
	Culture supernatant	Purified gassercin A
L. lactis subsp. *cremoris* MG1363	1/8	IP
MG1363(pIL253-P32)	1/7	IP
MG1363(pGAI)	−	IP
L. gasseri JCM1131^T	1/8	1/7
JCM1131^T(pIL253-P32)	1/8	1/8
JCM1131^T(pGAI)	−	−

[a] −, not inhibited; IP, inhibited by 60% (vol/vol) 2-propanol.

AR10 locus (*bvi*) from *B. fibrisolvens* AR10 (18) (Fig. 1). *gaaA* is 57.5% similar to *bviA* (the structural gene of butyrivibriocin AR10). *gaaT* and *gaaE* are 52.2% and 52.4% similar to *bviB* and *bviC*, respectively, although the two putative transporter genes of *bvi* reside upstream of *bviA* (Fig. 1). BviE, a putative immunity protein (18), shows low amino acid sequence similarity to GaaD. We did not find genes corresponding to the putative two-component regulator genes (ORF1, encoding a response regulator-like protein, and ORF2, encoding a histidine kinase-like protein), *bviD* (encoding a putative transporter element or immunity peptide), and ORF8 in the *bvi* locus detected in the 20-kbp sequence of LA39, while genes like *gaaB* and *gaaC* were not present in the reported *bvi* sequence. The production of several bacteriocins is regulated by pheromones (inducer peptides) and two-component regulator systems that respond to the pheromone (4, 9, 27). LA39 produces gassericin A constitutively when grown in MRS or milk-based media under various pH conditions (unpublished results). The lack of genes for a response regulator and sensor histidine kinase in the *gaa* locus may explain the observed nonregulated expression of gassericin A.

We studied the possibility that GaaI is the gassericin A immunity peptide. *gaaI* was cloned downstream of the lactococcal P32 promoter in pIL253-P32, and the heterologous expression and homologous expression of GaaI from the resulting plasmid, pGAI (Fig. 2), were examined in *L. lactis* subsp. *cremoris* MG1363 and *L. gasseri* JCM1131^T, respectively. Although the growth of *L. lactis* subsp. *cremoris* MG1363 was completely inhibited by 60% (vol/vol) 2-propanol to dissolve

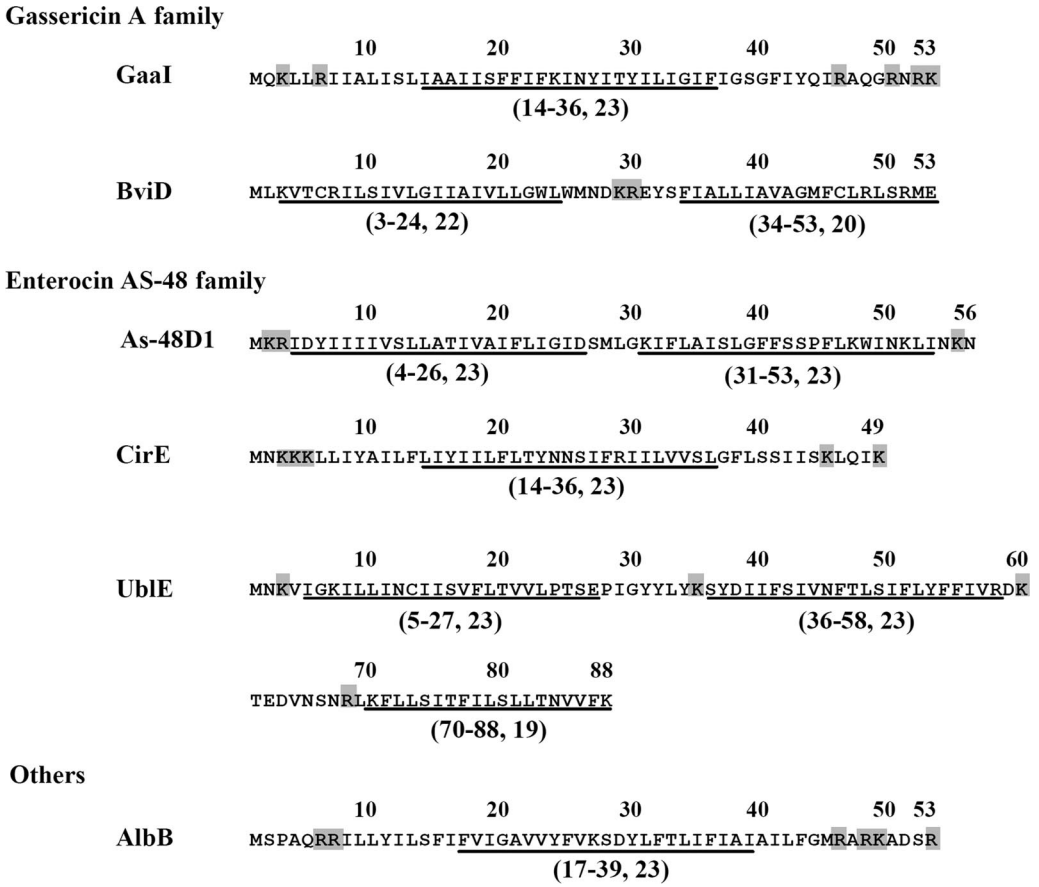

FIG. 3. Primary sequences of (putative) immunity peptides of circular bacteriocins. GaaI, BviD (putative; although BviD does not show homology to GaaI, the two peptides are the same length at 53 amino acid residues), As-48D1, CirE, UblE (putative), and AblB are the immunity peptides for gassericin A, butyrivibriocin AR10, enterocin AS-48, circularin A, uberolysin, and subtilosin A, respectively. The transmembrane regions deduced using the SOSUI program are underlined. The residue positions and lengths of the regions are shown within parentheses. Positively charged amino acid residues (lysine and arginine) outside of the TMSs are shaded.

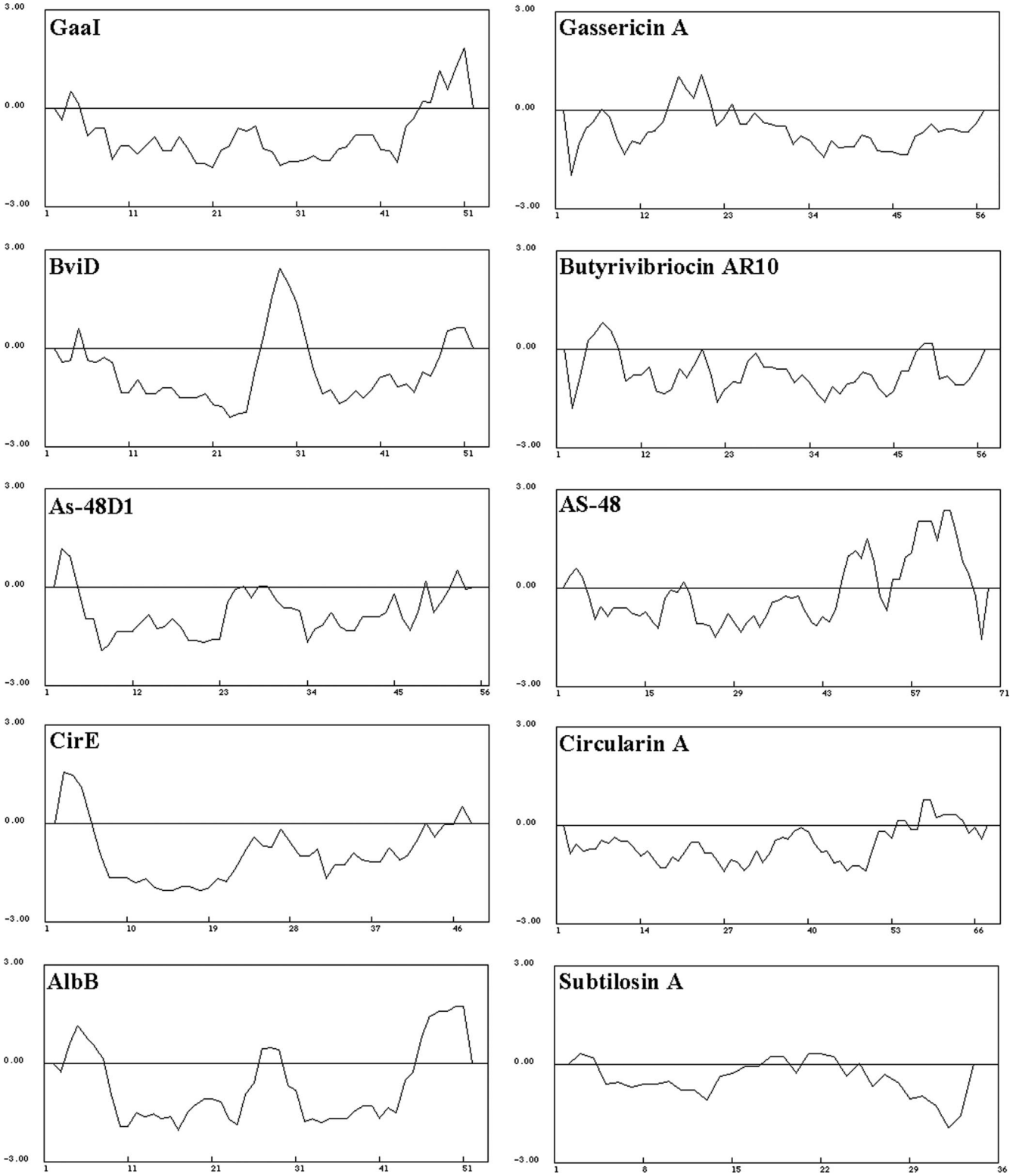

FIG. 4. Hydropathy of (putative) immunity peptides and cognate circular bacteriocins. Hydrophilic and hydrophobic regions were deduced using GENETYX-MAC software (Hoop and Woods [15]). The *x* axis shows the amino acid residue positions of the peptides, and the *y* axis is the hydropathy index.

the hydrophobic purified gassericin A for the bacteriocin tolerance assay, both recombinant strains were at least seven times more resistant to the purified gassericin A and the MRS broth culture supernatants of *L. gasseri* LA39 than the respective control strains carrying pIL253-P32 (Table 3). This indicates that *gaaI* encodes an immunity peptide for gassericin A. As shown above, the sequenced region of the *acd* locus is highly similar to that of *gaa*. However, the supposed homolog

of *gaaI* in the *acd* sequence may be completely disrupted (Fig. 1). This is unexpected, because *gaaI* may be the immunity determinant of *gaa*. If the *acd* cluster extended downstream of the reported sequence and contained homologs of *gaaT* and *gaaE*, these regions might constitute the primary resistance mechanism and would likely be the active export mechanism of acidocin B.

Usually bacteriocin producers also synthesize a protein(s) or peptide(s) to protect themselves from the actions of their own bacteriocins. ABC transporters, often with so-called accessory proteins, transport a cognate bacteriocin(s) and provide resistance by pumping out (active export) the bacteriocin molecules that have invaded from outside of the producing cells (4, 7). Many bacteriocin producers also make specific immunity proteins (peptides) to counteract the activity of the bacteriocin they produce. Generally, immunity proteins (peptides) are diverse in their primary structures (10, 34). Several small peptides, such as GaaI, have been implicated in providing immunity to circular bacteriocins (described below). Figure 3 shows the primary sequence and deduced transmembrane regions of the small immunity peptides for the class IV circular bacteriocins. The peptides, GaaI, BviD, As-48D1 (for enterocin AS-48), CirE (for circularin A), and AblB (for subtilosin A), ranging in size from 49 to 56 amino acid residues, have one or two transmembrane segments (TMSs), except for UblE (three TMSs), the putative immunity peptide for uberolysin, which consists of 89 amino acid residues and is slightly longer than the immunity peptides of other class IV bacteriocins (30). These TMSs may expose positively charged amino acid residues to the outer surface of the cytoplasmic membrane. These predictions suggest that the immunity peptides for class IV bacteriocins may be located in the cell membranes of the producing strains. As seen in Fig. 3, these peptides contain several lysines and arginines outside of the putative TMSs. This suggests that these positively charged residues on the peptides located on the outer and/or inner surface of the cell membrane may electrostatically prevent the attachment of cognate bacteriocins that are also positively charged. Conversely, many immunity proteins of the class IIa bacteriocins, ranging in size from 81 to 115 amino acid residues, which have been classified into three groups according to sequence homology (leucocin A and enterocin A in group A, piscicolin 126 and sakacin P in group B, and carnobacteriocin B2 and enterocin P in group C) (10), have no deduced TMSs and may be located at the inner surface of the cell membrane and act intracellularly (6, 10, 34). The mechanism of immunity peptides working against class IV bacteriocins could be different from that of immunity peptides of class IIa bacteriocins (8, 34, 38), as the modes of action of the two classes of bacteriocins may be different.

Figure 4 shows the hydrophobicity/hydrophilicity of the immunity peptides and the class IV bacteriocins predicted using the GENETYX-MAC software (Hoop and Woods [15]). The N and C termini and the middle region in the immunity peptides are hydrophilic and/or neutral, and the height of the hydrophilicity and the depth of the hydrophobicity are more than those of corresponding bacteriocins, except for AS-48. The orientation of hydrophobicity has nearby symmetry on a vertical line at the center of the peptides in spite of the different numbers of α-helical transmembrane regions (Fig. 3). This suggests that the immunity function works on the inner and outer cytoplasmic membranes against the compact globular class IV bacteriocins that are considered to invade on the intracellular side of the producing strains. We will require further study to unravel the intricacies in the mode of action and protection against the circular bacteriocins.

We attempted to produce gassericin A using the expression vector pIL253-P32 and *gaa* genes identified in this study. However, cloning of all seven *gaa* genes into pIL253-P32 was not successful in either *L. lactis* subsp. *cremoris* or *L. gasseri* hosts. No transformants of *L. gasseri* JCM1131^T were obtained with a pIL253-P32-based plasmid carrying the six consecutive ORFs *gaaCADITE* constructed in *L. lactis* subsp. *cremoris* MG1363. The five consecutive ORFs *gaaADITE* could be cloned in pIL253-P32 and introduced into *L. gasseri* JCM1131^T; however, no bacteriocin activity was detected in the culture supernatants of the recombinant strains (data not shown). Recently, an experiment to clone the seven *gaa* genes into another vector to express active gassericin A succeeded (data not shown). These results indicate that *gaaB* and/or *gaaC* with each promoter may be involved in the expression of active gassericin A. Further analysis and characterization of the expressed gassericin A from *L. gasseri* JCM1131^T are in progress. Although the nearly 100 naturally occurring circular proteins and peptides have been found in bacteria, plants, and animals, except for humans, and the circular structure is usually involved to provide stability against pH, heat, and proteolytic degradation, the cyclization mechanism for the involved enzymes and their auxiliary proteins remains to be elucidated (5). Circular bacteriocins, such as gassericin A and AS-48, of which the linear precursor peptide is encoded by one gene, should be good candidates for analysis of those seamless peptides.

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